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We have used the equine infectious anemia virus (EIAV)-Shetland pony system to examine the antigenic determinants, mechanisms, and in vitro serological correlates of vaccine enhancement of virus replication and disease, as an animal model for human AIDS vaccine studies. The results of these studies demonstrated that the propensity for vaccine enhancement depended on the nature of the envelope subunit immunogen, the antigenic relatedness of the vaccine and challenge strain, and individual differences in a typical outbred population of ponies. The current studies combined with previous vaccine trials indicated that vaccine enhancement was a practical problem with all of the experimental envelope subunit vaccines in challenges with heterologous virus, even in cases where the particular vaccine provided apparently sterile protection against homologous virus challenge. Evaluations of various serological assays of immune serum from vaccinated ponies demonstrated that in vitro assays were unreliable and insensitive correlates for immune enhancement in vivo, although antibodies associated with protective and nonprotective immune responses could be differentiated by serological properties that reflect the maturity of antibody responses to viral envelope proteins. Thus, these observations made in the EIAV system indicate a continued necessity to consider the potential for immune enhancement in the design of AIDS vaccine trials and to develop in vitro serological assays to monitor the maturation of antibody responses to candidate vaccines.

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R C Montelaro 4/30/97

PI - Signature Date

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A. INTRODUCTION

The potential for vaccine-induced enhancement of infections by enveloped viruses has been documented in both human and veterinary vaccine studies (6, 22, 27, 29). In the case of AIDS vaccine research, there has been considerable concern about the possibility of immune enhancement of HIV-1 replication in target lymphocytes and macrophage cells by various mechanisms, including classic antibody dependent enhancement (ADE) or increases in the number or activation levels of target cells by vaccination (2, 3, 13, 20). Several studies have demonstrated that serum antibodies from HIV-1 infected patients and SIV-infected monkeys can mediate ADE of virus replication in defined *in vitro* assays (9, 17, 18, 23), and limited studies have suggested that the level of ADE may correlate with the progression of disease (8). However, the significance of the observed *in vitro* ADE to the levels of virus replication *in vivo* remains uncertain and controversial. Moreover, the lack of clear *in vitro* correlates for *in vivo* ADE poses a risk to proposed AIDS vaccine trials in that there are no predictive assays for immune response that may result in either an increased susceptibility to infection by HIV-1 or acceleration of virus replication and disease upon infection by the virus. These questions are important to the U.S. Army in that HIV-1 exposure constitutes a clear threat to military personnel, especially in developing countries where AIDS is epidemic. Thus, the U.S. Army has established itself as a leader in the development of an effective AIDS vaccine.

Various animal lentiviruses have been used during the past 10 years as models for AIDS vaccine development and for evaluating potential immune correlates of vaccine protection or enhancement. We have been using the equine infectious anemia virus (EIAV) system as a model for natural immunologic control of a lentivirus infection and as a model to evaluate selected vaccine strategies against lentiviruses. In the course of evaluating various whole virus and subunit EIAV vaccines, we have observed a remarkable spectrum of vaccine efficacy, ranging from sterile protection with attenuated and inactivated whole virus vaccines to severe enhancement of EIAV replication and disease by a baculovirus expressed surface glycoprotein vaccine (rgp90) (11, 28). Earlier studies with CAEV (11, 15, 16, 28) and visna virus (21) reported vaccine enhancement in these animal lentiviruses, but the enhancement was predominantly defined by slightly higher levels of virus replication with no clear correlation to exacerbation of disease. More recently, vaccine enhancement has also been reported in vaccine trials using SIV (5, 24) and FIV (10, 26), again displayed primarily as slightly higher levels of virus replication in immunized animals compared to naive controls.

Based on our previous studies, the EIAV system appears to offer a potentially informative model in which to study vaccine enhancement in that immunization of ponies with the rgp90 vaccine resulted in dramatic increases in virus replication and severity of disease symptoms (28). Thus the EIAV model provides a uniquely relevant lentivirus system in which to examine the

mechanisms of vaccine enhancement, to evaluate the accuracy of *in vitro* assays as correlates of *in vivo* enhancement, and to define vaccine strategies that minimize the potential for eliciting enhancement.

The original two year specific aims of this research proposal were:

(1) To determine the extent of vaccine enhancement by rgp90 immunization in a larger vaccine trial and to assess if prior immunization with rgp90 enhances susceptibility to infection by homologous and heterologous strains of virus.

(2) To determine if passive serum transfers from rgp90 immunized ponies to naive ponies can elicit enhancement of EIAV replication and/or disease by challenges with homologous and heterologous virus strains.

(3) To evaluate whether a more rigorous immunization regimen can eliminate the enhancement observed with the standard rgp90 immunization protocol.

(4) To evaluate in pony vaccine trials the protective or enhancing properties of selected subunit vaccines, including individual viral glycoproteins and other baculovirus recombinant envelope immunogens.

(5) To examine the validity of *in vitro* ADE assays to predict *in vivo* enhancement and to use selected *in vitro* assays to elucidate the mechanisms and determinants of ADE in the EIAV system.

B. PROGRESS REPORT

The progress report for the grant period of July 15, 1994 to January 14, 1997 is organized according to the specific aims of the original grant proposal, and modifications in the research goal are discussed where appropriate:

Specific Aim #1. To determine the extent of vaccine enhancement by rgp90 immunization in a larger vaccine trial and to assess if prior immunization with rgp90 enhances susceptibility to infection by homologous and heterologous strains of virus.

We initially immunized 10 ponies at three week intervals with three 200 ug doses of the EIAV rgp90 vaccine in Syntex MDP adjuvant, as described previously (28). Two weeks after the last immunization, large volumes of plasma were collected from four of the vaccinated ponies for use in passive serum transfer experiments (Specific aim #3 below). At three weeks post immunization, the rgp90 vaccinated ponies were inoculated with 300 pony i.d. of our standard virulent virus strain, $EIAV_{PV}$. Two naive ponies were also challenged with PV to serve as controls. Ponies were monitored daily over a two month observation period for clinical symptoms (fever, diarrhea, edema, lethargy, etc.), and blood samples were taken daily for the first month and weekly thereafter for subsequent analyses of thrombocytopenia (which we have found a useful measure of in vivo enhancement), viral replication, and antibody responses.

Serological responses to the rgp90 vaccine and virus challenge. The EIAV envelope-specific antibody response to rgp90 immunization was monitored by Con A ELISA using viral glycoproteins or rgp90 in parallel to analyze serum samples taken on the day of challenge. As summarized in Figure 1 and Table 1, immunization with the rgp90 vaccine elicited relatively high levels of antibodies to the vaccine immunogen, with end point titers to rgp90 of 1/25,600 to 1/51,200 in nine of the ten vaccinated ponies and 1/12,800 in a single pony. In contrast, the levels of serum antibody reactivity with the native viral glycoproteins was on the average about 10-fold less than the reactivity observed against the rgp90 vaccine antigen. Serum end point titers with the viral glycoproteins ranged from 1/1,600 to 1/6,400 in nine of the ten vaccine recipients. This pattern of serum antibody reactivity demonstrates that the antibodies elicited by the rgp90 immunization react preferentially with the vaccine antigen and by comparison react relatively poorly with the native viral envelope proteins, as described previously.

Seroconversion to EIAV p26 after virus challenge was monitored by standard AGID and ELISA assays of serum samples taken on a daily basis (data not shown). As expected, all ponies were seronegative for EIAV p26 on the day of challenge. After inoculation with $EIAV_{PV}$, all ten of the immunized ponies became seropositive at 21-23 dpi, as observed with the control infections. Thus, the rgp90 vaccine failed to protect against EIAV infection and did not alter the rate of p26 seroconversion.

Clinical responses of ponies. To provide a more comprehensive data base for the pattern of clinical responses in naive ponies experimentally infected with a standard dose of $EIAV_{PV}$ (300-1000 $TCID_{50}$), we combined the two control ponies (#49, #87) from this experiment into a panel of 10 experimentally infected ponies (Table 2). Clinical responses were based on clinical impressions derived from daily observations to ascertain the severity of EIAV symptoms (fever, lethargy, diarrhea, edema, etc) and measurements of blood platelets which has proven to be a more informative measure of EIA disease than anemia. These data indicate that the first EIA febrile episode in experimentally infected ponies was generally observed (7/10 ponies) from 17-22 days post infection (dpi), with a mean of 18.8 days. Two ponies had no febrile episodes during the 60 day observation period, and one pony had its first fever at 35 dpi. Thrombocytopenia (defined as $\leq$100,000 platelets/ul) was not evident during the first febrile episode in any of the 10 control ponies, and a significant reduction in platelets was observed in only 3/10 infected ponies during their second febrile episodes at 34-39 dpi. Interestingly, the two ponies (#49, #87) used for control infections in the current rgp90 vaccine study represent the range of clinical responses observed with experimental infections with $EIAV_{PV}$; pony #35 experienced average clinical symptoms, while pony #49 experienced one of the most severe clinical responses that we have observed with this standard inoculation of naive outbred ponies with $EIAV_{PV}$.

In contrast to these control infections, a variety of responses was observed in rgp90-immunized ponies challenged with $EIAV_{PV}$ (Figure 2 and Table 2). Of the ten rgp90-immunized ponies challenged with $EIAV_{PV}$, four (#526,#66, #90, and #65) displayed more severe clinical symptoms of EIA compared to control infections, including earlier and more severe platelet declines associated with their initial febrile episodes at 12-23 dpi and depressed clinical attitudes. In fact, three of these four ponies had to be euthanized at 26-40 days post infection because of the severity of their clinical symptoms. Thus, these data indicate that the rgp90 immunization resulted in severe enhancement of EIA clinical symptoms in 40% of the vaccine recipients.

Four of the remaining six vaccinated ponies displayed clinical responses that appeared within the range observed with the panel of control experimental infections of naive ponies. For example, two vaccinated ponies (#120 and #118) also experienced a severe reduction in platelets associated with their first febrile episode, however, these initial episodes were at 36-37 dpi, at least

2 weeks later than the appearance of fever in the four ponies described above. Thus, these ponies appear to have a clinical response that is similar to the minority of experimental EIAV infections of naive ponies in which thrombocytopenia is observed concomitant with the first febrile episode. Two ponies (#64 and #60) experienced febrile episodes at 37 and 31 dpi, respectively, in the absence of a significant decline in platelets, which is typical of the clinical responses observed in the majority of control infections.

In contrast to the eight ponies described above that developed clinical symptoms of EIA, two ponies (#73 and #56) remained asymptomatic over the 45 day observation period, without detectable fever or clinical platelet declines. These observations suggest that the ponies were protected from the development of EIA disease by immunization with the rgp90 vaccine, although they were not protected from infection, as evidenced by p26 seroconversion.

In summary, 40% of the rgp90 vaccinated ponies displayed enhanced clinical symptoms, 40% had clinical responses that fell within the range observed for control infections, and 20% remained asymptomatic over the observation period, suggesting a degree of vaccine protection.

Pathologic gross and microscopic findings. To further characterize the severity of EIA disease in rgp90-vaccinated ponies, necropsies were performed for gross and microscopic pathology on selected individuals on the basis of their clinical signs compared to control virus infections: three immunized ponies with enhanced clinical symptoms (#526, #90 and #66), three immunized ponies with clinical EIA within the normal range, and one of the naive ponies (#49) infected with $EIAV_{PV}$ (Table 3). Among these ponies, the rgp90-immunized ponies #526 and #66 displayed the most diverse and severe pathological findings. The predominant gross findings were petechial hemorrhages on mucosal and/or serosal surfaces and remarkable histopathologic lesions characteristic of acute EIA, including the presence of pigment-laden macrophages in blood vessels. In addition, blood from pony #66 failed to clot, demonstrating further defects in clotting function in this pony whose platelet count immediately before euthanasia was 35,000/ul, the lowest among all of the ponies in this study. Less severe pathologic findings were observed in immunized ponies #60 and #120, primarily mild periportal lymphoplasmacytic infiltrates in the liver. In contrast, immunized pony #73, which remained asymptomatic after virus challenge, displayed only a very mild liver infiltration resulting from virus infection; no other gross or microscopic findings were evident.

Measurements of virus replication. The kinetics of EIAV replication in the ten rgp90-immunized ponies and two control ponies following virus challenge was initially followed by daily measurements of plasma viremia during the first 21 dpi., as described previously (28). These early assays for virus replication are designed to monitor the progression of virus replication prior

to EIAV p26 seroconversion, and thus to reflect the effect of vaccine immune responses on early stages of virus replication. The results of these plasma viremia assays are summarized in Table 2 and depicted graphically in Figure 2. The data demonstrate that a positive plasma viremia was first detected in the two infected control ponies at 11 dpi (#49) and 13 dpi (#87), respectively, and that the two ponies remained positive for plasma viremia for the remainder of the 21 day observation period, indicating persistent virus replication. As summarized in Table 1, the pattern of plasma viremia observed in the two control ponies used in this experiment was typical for other experimentally infected ponies.

The development of plasma viremia in the rgp90 immunized ponies indicated different patterns of virus replication among the vaccine recipients challenged with $EIAV_{PV}$. The four ponies that displayed enhanced clinical symptoms of EIA (#526,#90,#66,#65) became plasma viremia positive at 10-13 dpi and remained positive for infectious virus in plasma for the remainder of the 21 day observation period. Thus, this pattern of virus replication as measured by plasma viremia was similar to that observed in naive ponies inoculated with $EIAV_{PV}$. In contrast, the four immunized ponies that experienced a normal range of clinical symptoms (#120, #118, #64, #60) were substantially delayed in the appearance of detectable plasma viremia (first positive at 20-37 dpi) compared to control infections, suggesting an initial temporary immune control of virus replication by the rgp90 vaccine. Finally, the two rgp90 immunized ponies that remained asymptomatic during the observation period displayed markedly different patterns of plasma viremia. Pony #56 remained plasma viremia negative for the entire observation period, while pony #73 became plasma viremia positive at 12 dpi.

Taken together, the above plasma viremia data fails to establish a definitive correlation between the appearance of detectable plasma viremia and the observed severity of EIA in the immunized ponies. Thus, these results contrast with the previous rgp90 vaccine study in which disease enhancement was associated with an acceleration of virus replication, as seen by a more rapid appearance of plasma viremia and higher virus titers in immunized compared to control ponies infected in parallel with $EIAV_{PV}$. In the current study, the kinetics of plasma viremia between the immunized ponies with enhanced clinical symptoms were similar to control infections. However, the immunized ponies with enhanced clinical symptoms did, in general, appear to experience an accelerated development of plasma viremia compared to the rgp90 vaccinated ponies that experienced normal EIA symptoms or that remained asymptomatic. This latter comparison is compatible with a general relationship between the kinetics of virus replication and the severity of disease.

To provide a more quantitative measure of $EIAV_{PV}$ replication, a semi-quantitative RT-PCR was developed and used to measure EIAV-specific plasma RNA levels at 7, 14, and 21 dpi (Table 4). The results of these assays indicate that all of the ponies were negative for plasma viral

RNA at 7 dpi. Both of the infected control ponies had plasma RNA levels of 4 x 10^3 copies per ml at 14 dpi and 2-5 x 10^5 copies of viral RNA per ml at 21 dpi. Compared to these control infections, the four immunized ponies that experienced enhanced clinical symptoms had plasma viremia levels that ranged from about 10^4 - 10^7 RNA copies per ml at 14 dpi and 10^7 - 10^8 copies of RNA per ml at 21 dpi. These data indicate that clinical enhancement in the immunized ponies was associated with levels of virus replication that were on average 100-1000-fold higher than the viral RNA levels observed at similar time points in the control ponies. In contrast, the level of plasma viral RNA in the four immunized ponies that experienced typical EIA symptoms ranged from $<10^2$ -10^4 copies per ml at 14 dpi and $<10^2$ - 10^5 copies per ml at 21 days post infection. These data indicate a level of virus replication in this group of immunized ponies that was on average lower than that observed in the two control infections. Finally, the two immunized ponies that remained asymptomatic displayed markedly different levels of plasma viral RNA. Pony #56 viral RNA levels were relatively low at both time points, $<10^2$ at 14 dpi and 9 x 10^2 at 21 dpi, while pony #73 RNA levels were measured at 3 x 10^6 at 14 dpi and 1x 10^6 copies/ml at 21 dpi.

These quantitative plasma RNA data presented here indicate a general correlation between the severity of disease and the levels of virus replication in the panel of immunized ponies; ponies experiencing enhanced clinical symptoms had plasma RNA levels that were at 21 dpi an average 10^4-fold higher compared to the group of immunized ponies that showed normal EIA symptoms and 10^2-fold greater than the plasma RNA levels observed in the control pony infections. However, the correlation between disease severity and virus replication levels is not absolute, as immunized pony #73 remained asymptomatic with plasma RNA levels that were 10-fold greater than observed in the control pony infections.

For comparison, we also used the newly developed semi-quantitative RT-PCR to assay the levels of EIAV RNA in plasma samples taken from four ponies that were immunized with rgp90 in the 1991 vaccine trial and that displayed severe enhancement. These data (Table 4) indicate that the four challenged ponies had negligible levels of viral RNA in plasma at 7 dpi, 10^6-10^8 copies/ml at 14 and 21 dpi. Thus, these plasma RNA measurements are consistent with the previous observations of accelerated viremia in the rgp90 vaccine recipients challenged with $EIAV_{PV}$.

Comparison of 1991 and 1995 rgp90 vaccine trials. In the previous rgp90 vaccine trial, we observed enhancement of clinical symptoms, accelerated viremia, and markedly increased levels of virus replication in 4 of 4 immunized ponies challenged with $EIAV_{PV}$ (28). In this current rgp90 vaccine trial using an identical immunization regimen, we observed a spectrum of clinical responses that ranged from severe enhancement to protection from the development of disease. Initially, we were concerned that the differences observed between the two vaccine trials might be the result of small changes in detergent composition that were made in the preparation of the

recombinant rgp90 immunogen to provide an antigen solution that was more stable in terms of solubility upon storage. The hypothesis was that the alteration in immunogen might have resulted in a modified immune response, a possibility that was suggested by some initial comparative serology performed on the two panels of rgp90-immunized ponies (c.f. Table 1). Thus, we immunized 2 pairs of ponies with 4 and 5 doses, respectively, of the most recent rgp90 vaccine to see if a more rigorous immunization would change the serological properties of the immune responses and the clinical pattern observed after standard virus challenge. The results of this experiment demonstrated that additional immunizations did in fact alter serological properties of the EIAV-specific antibodies, but did not affect the levels of enhancement after virus challenge; only 1 of four ponies displayed enhanced clinical symptoms (described in Specific Aim #3 below). As an alternative strategy, we subsequently prepared another batch of rgp90 vaccine using the original detergent compositions used in our first rgp90 vaccine trial. Once again, four ponies were administered 3 doses of this rgp90 vaccine and subjected to the standard virus challenge. In this last vaccine trial, 1 of 4 ponies displayed enhanced clinical symptoms. Taken together, these experiments suggest that the differences observed in extent of vaccine enhancement were probably not due to the nature of the rgp90 protein used in the vaccine.

Modifications of research plan for Specific Aim #1. A second component of this specific aim was to determine if rgp90 immunization increased the susceptibility of ponies to infection with EIAV. While this experiment seemed feasible with a vaccine enhancement rate of 100%, the observations of a substantially lower rate of vaccine enhancement in a larger population of ponies made this objective impractical. Therefore, it was determined in consultation with our grant supervisors to not pursue this aspect of Specific Aim #1, as the number of ponies required to provide a statistically significant result was prohibitive.

Conclusions from Specific Aim #1. As expected, the rgp90 vaccine failed to protect ponies from infection by experimental heterologous challenge with $EIAV_{PV}$. The results from the above vaccine trials demonstrated a spectrum of rgp90 vaccine efficacy, ranging from severe clinical enhancement of EIA symptoms in up to 40% of vaccine recipients to protection from the development of clinical EIA in about 20% of vaccine recipients. The severity of disease observed in immunized ponies in general correlated with the levels of virus replication (as measured by PCR analyses of plasma RNA), but this correlation was not absolute. Overall, immunized ponies with enhanced clinical symptoms displayed levels of virus replication that were 10^2-10^3-fold higher than the naive control ponies infected with $EIAV_{PV}$. These observations suggest that rgp90 immunization can result in a previously unrecognized range of effects on clinical outcome and virus replication in a population of vaccinated ponies. However, these studies also confirm the potential for vaccine enhancement

of EIAV replication and disease at a frequency that would pose a serious obstacle to any vaccine effort.

Specific Aim #2. To determine if passive serum transfers from rgp90 immunized ponies to naive ponies can elicit enhancement of EIAV replication and/or disease by challenges with homologous and heterologous virus strains.

As a preliminary passive transfer experiment, we used a mare (#102) and her foal (#102F) as a donor and recipient combination to assess the ability of passively transferred plasma from a rgp90 vaccinated pony to mediate enhancement in a donor pony. Thus, pony #102 was immunized as usual with the rgp90 vaccine (3 doses at monthly intervals). One day prior to virus challenge, 500 ml of immune plasma was collected from the donor mare #101. The following day it was administered over a 2 hr period to the recipient foal. Immediately after the transfer, the foal and the mare were challenged with the standard $EIAV_{PV}$ inoculum and monitored daily for clinical symptoms of EIA. As summarized in Figure 3, the donor and recipient pony demonstrated the similar enhanced clinical profiles, i.e., thrombocytopenia associated with first fever by 21 dpi. These results indicated that immune enhancement of EIA disease could be accomplished in a naive pony by passive transfer of plasma from a rgp90-vaccinated donor pony.

Based on these encouraging initial observations, we next collected large volumes of plasma from rgp90-immunized ponies #526 and #56 described in Specific Aim #1 above (c.f. Fig. 2) prior to experimental virus challenge. Interestingly, pony #526 displayed severe enhancement of clinical symptoms after virus challenge, while pony #56 remained asymptomatic. Thus, the two plasma samples fortuitously represented opposite ends of the rgp90 vaccine efficacy spectrum, apparently offering an opportunity to determine if both enhancement and protection could be passively transferred. As described above, 500 ml of immune plasma from each donor was administered over a 1 hr period to a single recipient pony: pony #526 -> #582 and pony #56 -> #584. Immediately upon completion of the plasma transfer, the recipient ponies were challenged with the standard $EIAV_{PV}$ inoculum and monitored daily for clinical symptoms of EIA. As summarized in Figure 4, both ponies became infected with EIAV and developed plasma viremia by about 13 dpi. However, the clinical profiles of the two recipient ponies differed markedly. Pony #582 that received the "enhancing" #526 plasma experienced a severe thrombocytopenia coincidentally with development of the first febrile episode, as observed with enhanced clinical responses with rgp90 vaccinated ponies, including the donor pony #526. In contrast, pony #584 ("protective" #56 plasma recipient) experienced only a minor reduction in platelets associated with an initial mild febrile period; thrombocytopenia was associated with subsequent relatively mild febrile episodes. This latter clinical pattern is characteristic of EIA resulting from experimental infections of naive ponies in the development of thrombocytopenia, although the passively transferred antibodies may have moderated fever and other clinical symptoms in this recipient pony.

Conclusions from Specific Aim #2. The results of the experiments demonstrated a degree of clinical enhancement in recipient ponies receiving plasma from rgp90-immunized donor ponies that had also experienced enhancement after EIAV challenge. It remains to be determined if the observed clinical enhancement is associated with an enhancement of virus replication, as determined by RT-PCR analysis of plasma viral RNA levels. The enhancement observed in the recipient ponies in general resembled the pattern of enhancement observed in the respective donors, i.e., severe clinical impressions (lethargy, diarrhea, etc.) and development of thrombocytopenia with the first febrile episode. Interestingly, the "protective" plasma from a rgp90-vaccinated pony that remained asymptomatic after virus challenge failed to provide complete protection from the development of disease symptoms in the recipient pony after virus challenge, although it did experiments in our lab in which passive immune serum from ponies immunized with vaccines that provide sterile protection against virus challenge (e.g., inactivated whole virus) can at best delay or moderate the development of disease in recipient ponies. In fact, passive transfer experiments have in general met with only limited success in several lentivirus systems, including SIV/macaque and FIV/cat (20).

While the limited data here is compatible with humoral factors mediating clinical enhancement, it should be emphasized that additional experiments need to be performed to establish a definitive correlation. We anticipate performing several more passive transfer experiments to develop a more comprehensive analysis of the effects of rgp90 immune serum on clinical progression and virus replication in recipient ponies challenged with $EIAV_{PV}$.

Specific aim #3. To evaluate whether a more rigorous immunization regimen can eliminate the enhancement observed with the standard rgp90 immunization protocol.

As noted above, we have recently described a panel of serological assays that can be used to elucidate an evolution of antibody responses to lentivirus infections (EIAV, SIV, HIV-1) and to define immature and mature immune responses that correlate with the development of broadly protective immunity in the case of SIV (4). Initial serological analyses of envelope-specific antibodies elicited by the rgp90 vaccine in the current (1995) study indicated characteristics of an immature immune response, i.e., low reactivity with native viral envelope protein, low conformational dependence, and low avidity (c.f. Table 1 and Specific Aim #5 below). Similar serological analyses of the antibodies elicited by rgp90 immunization in the 1991 vaccine trial indicated similar in vitro properties, except that the antibody titers to rgp90 and the viral glycoproteins were at least 10-fold less than observed in the 1995 vaccine trial (Table 1).

Therefore, we wished to determine if a more rigorous immunization with rgp90 would change the properties of the antibody responses and the propensity for enhancement. For this experiment, we selected 6 ponies that had received the standard 3 dose rgp90 regimen in parallel with the 10-pony panel described in Specific aim #1 and administered one or two additional vaccine doses to two ponies each for a total of 4 and 5 immunizations, respectively. The serological properties of the antibody responses during the course of immunization were followed by measuring antibody reactivity with rgp90 and native viral envelope proteins in our standard ELISA assay. Three weeks after the final immunization, the vaccinated ponies were challenged with the standard $EIAV_{PV}$ inoculum and monitored daily for clinical symptoms and virus replication to determine the levels of protection or enhancement provided by the extended immunization regimen.

The results of the ELISA assays for antibody reactivity to rgp90 and to the viral glycoproteins (vgp) (Table 5 and Figure 5) indicate that the fourth and fifth immunizations in general preferentially increased the serum antibody reactivity with the rgp90 antigen substrate compared to the vgp antigen in parallel ELISA. For example, after the third immunization with rgp90, the reactivity titer was on average 2-fold greater with the rgp90 compared to the vgp antigen in the ELISA assay. After the fourth immunization, however, the serum reactivity to rgp90 was on average at least 10-fold greater compared to the vgp antigen, indicating a markedly greater specificity for the recombinant protein. This differential in antibody specificity for antigen was maintained after the fifth immunization.

While the additional vaccine doses altered the reactivity profile of the serum antibodies in the immunized ponies, the more rigorous immunization did not appear to substantially alter the clinical responses of these ponies when challenged with the standard $EIAV_{PV}$ inoculum. Two of

the six ponies displayed enhanced clinical symptoms, and the remaining four experienced typical clinical responses after virus challenge.

Changes in Specific Aim #3. The original research plan under this specific aim proposed to evaluate the effects of alternative adjuvants combined with the rgp90 immunogen as a vaccine protocol. In particular, we proposed to use Freunds complete adjuvant in the initial vaccine dose, followed by two doses with Freunds incomplete adjuvant. As an initial experiment, we first evaluated the effect of Freunds on a pony using the proposed immunization regimen. The results of this experiment indicated that the reactions at the site of administration of the Freunds adjuvant were too extensive and too extreme to be used in compliance with animal welfare standards. Therefore, these experiments were not pursued, and our efforts were focused more on evaluating the efficacy of EIAV vaccines based on different envelope immunogens, as described in Specific Aim #4.

Conclusions from Specific Aim #3. The results of this component of the research project demonstrated that the specificity of serum antibodies elicited by rgp90 vaccine was in fact dependent on the immunization regimen employed, with additional vaccine doses increasing the antibody titer and specificity for the rgp90 antigen compared to the viral envelope proteins as ELISA substrates. However, this change in antibody specificity evidently did not alter the frequency of vaccine enhancement in response to EIAV challenge.

Specific aim #4. To evaluate in pony vaccine trials the protective or enhancing properties of selected subunit vaccines, including individual viral glycoproteins and other baculovirus recombinant envelope immunogens.

As described in the Introduction section above, previous experimental vaccine trials in the EIAV system have demonstrated a wide range of efficacy depending on the nature of the vaccine immunogen. In light of the interest in subunit envelope antigens as a basis for AIDS vaccines, we sought to assess further the protective/enhancing properties of selected envelope subunit vaccines in the EIAV system, with a particular interest in comparing vaccines based on viral envelope proteins to baculovirus recombinant proteins. The results of these studies are summarized below.

(a) Vaccination with lentil lectin affinity purified viral envelope proteins (LL-gp) - We reported previously that immunization of ponies with lectin affinity-purified viral envelope proteins (gp90 and gp45) from $EIAV_{Pr}$ using the standard Syntex MDP adjuvant and three dose immunization regimen appeared to provide protection from infection after homologous virus challenge with $EIAV_{Pr}$, but resulted in vaccine efficacy ranging from protection to enhancement in response to heterologous virus challenge with $EIAV_{PV}$ (11). In this set of experiments we sought to extend these studies to determine the levels of protection and enhancement produced by immunization with a LL-gp vaccine derived from $EIAV_{PV}$ using a homologous virus challenge. Thus, four ponies were immunized with 3 doses of LL-gp (200 ug), challenged 3 weeks after the final immunization with 300 i.d. of $EIAV_{PV}$, and monitored daily for clinical responses and virus replication. As summarized in Figure 6, three of the four LL-gp-immunized ponies (#45, #95, and #100 remained asymptomatic (no detectable fever or other signs of EIA); pony #84 developed a slight fever at 12-16 dpi, but this fever was not associated with any other symptoms of EIA (e.g., diarrhea, lethargy, edema, etc.). Analyses of platelet counts in each of the ponies indicated that none of the ponies developed thrombocytopenia; three ponies showed no decline in platelets, while one pony had a slight drop in platelets concomitant with a mild fever. All four ponies remained negative for plasma viremia over the observation period and were shown to be negative for viral plasma RNA by RT-PCR at 7, 14, and 21 dpi (Table 5). These results demonstrate for the first time a high degree of vaccine protection against challenge with the virulent $EIAV_{PV}$, in distinct contrast to the lack of vaccine efficacy and enhancement observed with the rgp90 trials described above.

(b) Vaccination with viral gp90 (vgp90) - In light of the high levels of protection provided by the LL-gp vaccine, we next evaluated an experimental vaccine using viral gp90 protein isolated from gradient-purified $EIAV_{PR}$ by preparative HPLC, as described previously (1). This

vgp90 protein preparation has been shown to contain 99.5% gp90 and less than 0.5% p26, a practical limitation on purification in which 100 mg of virus are required to produce about 1 mg of vgp90. The vgp90 immunogen was mixed with the Syntex MDP adjuvant and administered to 6 ponies in 3 doses (10 ug each) following the standard immunization regimen. Three ponies each were then subjected to homologous challenge with $EIAV_{Pr}$ or to heterologous challenge with $EIAV_{PV}$ as described previously and monitored daily for clinical symptoms (Figure 7). The results demonstrate that all three of the $EIAV_{PV}$-challenged ponies developed initial febrile episodes at about 8 dpi. Two of the ponies (#412 and #449) displayed typical clinical impressions and had only a slight drop in platelets associated with the initial febrile episode. In contrast, one pony (#88) displayed severe clinical symptoms and severe thrombocytopenia (15 dpi) requiring euthanization at 26 dpi, a clinical response indicative of vaccine enhancement. Assays for plasma viremia (Fig. 7) indicated that ponies #412 and #449 experienced transient or intermittent plasma viremia during the first 21 dpi, while pony #88 became consistently plasma viremia positive at 8 dpi. Analyses of EIAV plasma RNA levels (Table 6) in these ponies indicated that the enhanced disease observed in pony #88 was associated with accelerated levels of virus replication compared to the other two ponies. At 14 and 21 dpi, pony #88 displayed plasma RNA levels of 5×10^6 and 3×10^7 copies per ml, respectively. The other two ponies challenged with $EIAV_{PV}$ displayed 14 dpi plasma RNA levels of 7×10^2 and 1×10^5 copies per ml, and 21 dpi levels of 1×10^3 and $<10^2$ copies per ml, respectively.

In contrast to the responses observed with the heterologous $EIAV_{PV}$ challenge, the 3 vgp90-immunized ponies subjected to homologous virus challenge with the avirulent $EIAV_{Pr}$ remained asymptomatic for EIA disease over the 60 day observation period; none of the ponies developed thrombocytopenia, although two ponies did develop mild fevers at 3-4 dpi. All three of these ponies remained plasma viremia negative for the initial 21 dpi and were PCR negative for plasma RNA at 7, 14, and 21 dpi. Thus, these data indicate that the vgp90 provided a high degree of protection against infection by homologous virus challenge. Whether or not this can be considered "sterile protection" will require PCR analyses of plasma RNA levels at later time points post infection.

(c) Vaccination with EIAV p26. In light of the fact that the LL-gp and vgp90 vaccines described above contained minor quantities of viral p26 that elicited antibody responses in the immunized ponies, we wanted as control experiments to evaluate the potential contribution of these p26 immune responses to protection or enhancement. Six ponies were immunized with three doses of purified recombinant p26 (100 ug per dose) in MDP adjuvant using standard protocols. Three weeks following the final immunization, three ponies each were subjected to standard challenge with either $EIAV_{Pr}$ or $EIAV_{PV}$ and monitored daily for the development of clinical

symptoms and plasma viremia. As controls, two ponies each were challenged with the standard $EIAV_{Pr}$ and $EIAV_{PV}$ inocula and monitored in parallel to the p26-immunized ponies. The results of these experiments indicated that p26 immunization failed to protect against infection by either strain of virus (all ponies were plasma viremia positive) and did not substantially affect the development of viremia or clinical disease compared to infection of control ponies (data not shown). These observations indicate the protection or enhancement observed with the LL-gp and vgp90 vaccines is likely due to the predominant envelope antigens and not to the minor quantities of p26 contained in these immunogen preparations.

(d) Conclusions from Specific Aim #5. The results of these studies demonstrate for the first time that an experimental vaccine based on EIAV envelope proteins (i.e., LL-gp and vgp90) can provide protection from infection and disease by homologous virus challenge. The EIAV $LL\text{-}gp_{PV}$ vaccine prevented establishment of infection and development of disease by challenge with 300 pony i.d. of the homologous $EIAV_{PV}$. Similarly, the $vgp90_{Pr}$ vaccine protected against the establishment of infection by challenge with about 10^3 pony i.d. of the homologous $EIAV_{Pr}$ strain. In contrast, previous studies (11) indicated that $LL\text{-}gp_{Pr}$ immunization could not protect against heterologous virus challenge, and the results of the current studies demonstrate that the $vgp90_{Pr}$ vaccine failed to protect against heterologous challenge with $EIAV_{PV}$. Thus, these data support the conclusion that vaccine protection against homologous virus is in fact more easily achieved than against heterologous virus challenge. In the case of the two EIAV virus strains used in these studies, there exist differences both in their pathogenic and antigenic properties. However, in light of the vaccine protection observed here against the various strains, we believe that the final vaccine efficacy is predominantly dependent on the antigenic properties of the viral envelopes between the two EIAV strains. In this regard, the $EIAV_{Pr}$ and $EIAV_{PV}$ envelope sequences differ by only about 2% in gp90 and <1% in gp45, suggesting that minor differences in amino acid sequences can result in major differences in antigenic properties and vaccine efficacy.

The second conclusion from these vaccine trials is that vaccine enhancement is most likely to occur with heterologous virus challenge, as observed with both the rgp90 and vgp90 vaccines. In the case of rgp90 vaccine, enhancement occurred in about 40% of the immunized ponies. In the case of the vgp90 vaccine, one of four immunized ponies displayed severe enhancement, "suggesting" an enhancement frequency of about 25%. This latter number is similar to the frequency of enhancement previously observed with heterologous virus challenge in LL-gp90 immunized ponies (11).

The third conclusion from these studies is that vaccine enhancement cannot be attributed solely to the use of recombinant viral antigens, but can also result from subunit vaccines based on viral envelope proteins, e.g., vgp90 (these studies) and LL-gp (Issel et. al. 1992).

Taken together, these observations recommend that candidate viral envelope subunit vaccines be based on antigens that closely maintain native multimeric structure and that match the antigenic properties of the predominant virus strains.

Specific Aim #5. To examine the validity of *in vitro* ADE assays to predict *in vivo* enhancement and to use selected *in vitro* assays to elucidate the mechanisms and determinants of ADE in the EIAV system.

(a) Development of in vitro enhancement assays.

During this project period, we have placed a high priority and major investment of effort into developing an in vitro enhancement assay that could be used to examine the mechanisms of immune enhancement and that could be evaluated as a correlate of in vivo enhancement. The success of this effort depended largely on the assumption that the observed vaccine enhancement of EIAV replication and disease was most likely mediated by ADE mechanisms (either complement dependent or independent) that could be reproduced in vitro with appropriate target cells, immune serum samples, and assay conditions. Thus, each of these parameters were exhaustively evaluated towards the development of an in vitro assay to measure ADE of EIAV replication.

(i) Cell targets. EIAV is believed to infect only cells in the monocyte/macrophage lineage, and in infected horses, the predominant sites of virus replication are tissues with abundant macrophage populations (e.g., spleen, liver, kidney, lung, etc.); there is only very limited infection of blood monocytes, despite the relatively high viremia levels evident during clinical episodes (19). As with other lentiviruses, EIAV replication in infected blood monocytes depends on activation and differentiation to macrophage (14, 25). Based on these observations and the relative availability of sources of macrophages from horses, we focused our initial efforts on developing procedures for efficient and reproducible isolation and in vitro cultivation of equine macrophages. Previous efforts to cultivate equine macrophages in even short term culture have met with only limited success, and we also found these published procedures to be relatively unreliable. During the past two years we have developed standard procedures for culturing equine macrophages isolated from tissues (12) or derived from equine PBMC (in preparation). The availability of these macrophage cultures for the first time allowed measurements of EIAV infectivity and antibody neutralization in the natural target cell for EIAV, rather than using various equine fibroblastic cell cultures or transformed feline or canine cell lines , as required previously. It should be noted here that the development of these equine macrophage culture procedures represent a major advance in EIAV research (although this may be hard to believe for investigators that work with human or mouse macrophages!) and that this advance was made possible by the resources provided by this research grant.

(ii) Optimization of conditions for ADE assay. We extensively evaluated a number of different conditions to maximize sensitivity and specificity of the in vitro enhancement assay, including conditions for treatment of virus inoculum with test immune serum (virus dose, serum dilutions, length of treatment, etc.), conditions for culturing cells after infection (with and without a dilution of the test immune serum), and methods for assaying levels of virus replication (RT vs PCR) and CPE (vital stains vs visual analysis). Based on these experiments, we observed that the maximum levels of serum enhancement of EIAV replication were observed when the standard virus inoculum (moi of 0.001) was first incubated with a 1:4 dilution of immune serum (taken on day of challenge) at 37° C for 1 hr and then allowed to infect the macrophage cells for two hours, after which time the cells were cultured in the continued presence of the test immune serum at a 1:45 dilution in media for up to 14 days after infection with daily measurements of virus production by daily assay of supernatant RT activity . Using these "optimum" reaction conditions to evaluate various panels of immune serum from rgp90-immunized ponies, we made the following observations:

(1) Are in vitro enhancement assays a reliable indicator of in vivo enhancement? Increased virus replication was only infrequently observed in the in vitro assay, even when immune serum was taken from ponies that had exhibited the most severe (even fatal) enhancement of EIAV replication and disease in vivo. Therefore, in our hands, in vitro enhancement must at best be considered a very insensitive and irreproducible measure of in vivo enhancement in the EIAV system.

(2) Is in vitro enhancement a reproducible observation when detected with a particular immune serum ? In a minority of experiments, in vitro enhancement was evident in that the levels of virus replication in cultures treated with a particular rgp90 immune serum were in fact 2-4-fold greater than that observed in parallel virus cultures treated with pre-immune serum from the same pony. However, this enhancement was seldom reproducible in repeated experiments.

(3) Can in vitro enhancement be mediated by purified Ig with or without complement? In vitro enhancement was only observed with immune serum; we never observed in vitro enhancement with purified Ig from the respective immune serum, suggesting the requirement of other serum components as essential co-factors in in vitro enhancement. We also were unable to demonstrate a role for equine complement in the in vitro enhancement.

(4) Can in vitro enhancement be mediated by immune plasma, rather than immune serum? Immune plasma taken from vaccinated ponies was no more effective in the in vitro enhancement assays than its respective immune serum; observed plasma enhancement was infrequent and then typically less than 2-fold over plasma taken from control naive ponies. Therefore, there does not appear to be enhancing components in immune plasma that might be absent in immune serum samples.

(5) Could enhancement be occurring by infection of monocytes rather than macrophage? To test this hypothesis, monocytes from a naive pony were infected in Teflon containers with the standard EIAV inocula treated with pre-immune serum or rgp90 immune serum, adhered to tissue culture plates overnight with the removal of nonadherent cells, and monitored daily for virus production. The results of these experiments demonstrated that replication of EIAV occurred in the infected monocytes after attachment, but that the levels of virus replication in the macrophage cultures were similar between the pre-immune and immune serum treatments. Thus, we found no evidence for immune serum enhancement of EIAV infection of monocytes in vitro.

(6) Does in vitro enhancement require autologous plasma or serum and macrophage? Assays of virus replication in macrophage cultures in the presence of autologous immune serum or plasma demonstrated patterns of in vitro enhancement similar to that observed using heterologous target cells and immune serum or plasma. The observed enhancement of virus replication by immune serum or plasma was infrequent and when evident less than 2-fold greater than pre-immune serum or plasma.

(b) Analyses of serological assays as correlates of immune enhancement. In addition to evaluating in vitro enhancement assays as a potential correlate of in vivo enhancement, we also examined a number of different serological assays for their ability to distinguish enhancing from protective immune responses. For these analyses, we used a combination of immunoassays that have proven useful in defining the maturation of antibody responses to EIAV (7) and SIV (4) infections and with the development of protective immunity. These serological assays included: antibody reactivity to native viral envelope proteins and vaccine immunogen, antibody conformational dependence (ratio of reactivity with native envelope compared to denatured envelope antigen), antibody avidity (resistance of serum antibody-envelope antigen complexes to urea treatment), and serum neutralization activity. Based on these serological assays, we have defined antibody properties of immature and mature antibody responses to lentivirus infection and suggested that successful vaccines must achieve the necessary level of immune maturation to provide protective immunity from virus challenge. We have also proposed that immature immune responses may actually enhance virus replication during the early stages of infection and after experimental challenge of vaccinated animals.

To test this hypothesis, we assayed immune serum from our panels of rgp90 vaccinated ponies to determine if any single or combination of serological parameters would be a reliable indicator of in vivo enhancement. In parallel, we also analyzed immune serum samples from ponies immunized with a lectin affinity purified EIAV envelope proteins (LL-gp) (see below) as a source of serum associated with a protective immune response that prevented infection after

experimental EIAV challenge. Representative results of these assays are summarized in Table 1 and Figure 8.

The results of these serological assays indicate that each of the vaccines elicit a characteristic antibody response. For example the rgp90 vaccine (1995 trial) produced antibodies that reacted well with the rgp90 antigen (avg. titer of 1/67,000) and poorly with viral glycoproteins (avg. titer of about 1/5,000) in ELISA, were predominantly directed to linear determinants on the viral envelope proteins (avg. conf. depend. of 0.7), low avidity (<5%), and lacking neutralization activity (titers <1/16). The LL-gp vaccine elicited antibodies that reacted preferentially with the viral envelope proteins compared to the rgp90 antigen (avg. titers of 1/102,400 and 1/6,400, respectively), were predominantly directed to conformational envelope determinants (avg. conf. depend. of 1.2), low avidity (<10%), and lacking neutralization activity. The vgp90 vaccine generated antibody responses that reacted fairly equally with viral envelope proteins compared to the rgp90 antigen, were predominantly directed to linear envelope determinants (avg. conf. depend. of 0.5), and that were low avidity, and poorly neutralizing. Thus, each of the vaccines could be differentiated by the panel of serological assays employed here.

Comparison of the serological properties of various vaccine antibody responses to those of immature and mature antibody response in EIAV-infected ponies indicate that none of the experimental immunizations accomplish a complete maturation of the immune response. The most protective and least enhancing vaccine, LL-gp, is characterized by antibody responses that most resemble the properties of the mature antibody response to EIAV envelope proteins with respect to antibody antigen specificity and conformational dependence, but not in avidity and neutralization activity. The least protective and most enhancing vaccine, rgp90, is characterized by vaccine antibody responses that most resemble those associated with immature immune responses to EIAV, including relatively poor reactivity with native viral antigens, low conformational dependence, low avidity, and lack of neutralization activity. The final vaccine, vgp90, which produced a mixture of protection and enhancement interestingly is associated with envelope-specific antibody responses are intermediate between those observed with immature and mature immune responses to EIAV.

Finally, the neutralization data described here indicate that the neutralizing antibodies to the challenge virus strain are not required for protection, as the homologous protection observed with the LL-gp and vgp90 vaccines was achieved in the absence of significant levels of neutralizing antibodies to the challenge virus. This result then is in agreement with our previous observations with EIAV inactivated whole virus and LL-gp vaccine trials in which protection was achieved in the absence of detectable neutralizing antibody to the challenge virus stock.

(c) Conclusions from Specific Aim #5. The major conclusions relative to the EIAV system that can be deduced from the studies described in this specific aim are:

(i) In vitro enhancement assays are at best insensitive and unreliable indicators of in vivo enhancement.

(ii) The properties of viral envelope-specific antibodies elicited by immunization are highly dependent on the nature of the vaccine immunogen.

(iii) Vaccine-associated antibody responses can be distinguished by a panel of serological assays that include antigen specificity, conformational dependence, avidity, and neutralization in vitro.

(iv) None of the individual serological parameters provide a reliable immune correlate of protection, including serum neutralization activity against the challenge stock.

(v) Vaccine antibody responses that provided a high degree of protection most closely resemble antibodies associated with mature immune responses to EIAV envelope proteins, while vaccines that had a high propensity for enhancement most resembled antibodies associated with early, immature responses to EIAV envelope proteins.

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D. PUBLICATIONS AND MEETING ABSTRACTS RELATED TO THIS GRANT.

Publications:

Montelaro, R., Grund, C., Raabe, M., Woodson, B., Cook, R., Cook, S., and Issel, C. (1995) Characterization of protective and enhancing immune responses to EIAV resulting from experimental vaccines (A brief review). AIDS Res. Human Retroviruses 12: 413-415.

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Montelaro, R., Cook, S., Woodson, B. and Issel, C. (1997) Efficacy of experimental viral envelope subunit vaccines against EIAV infection and disease range from sterile protection to severe enhancement. In preparation (to be submitted by June 30, 1997).

Raabe, M. and Montelaro, R. (1997) Development of equine macrophage cultures and their application to infectivity and neutralization assays for EIAV. In preparation (to be submitted by June 30, 1997).

Raabe, M., Grund, C., Woodson, B., Cook, S., Issel, C., and Montelaro, R. (1997) Evaluation of in vitro serological assays as indicators of protective and enhancing immune responses to EIAV elicited by experimental immunization. In preparation (to be submitted by July 31, 1997).

Note: Copies of manuscripts will be made available as accepted for publication.

Meeting Abstracts:

Montelaro, R. (1995) Protective and enhancing immune responses to EIAV in experimentally vaccinated ponies. International Mtg. on Comparative Retroviral Vaccines. Annecy, France.

Montelaro, R., Grund, C., Raabe, M., Cook, R., and Issel, C. (1995) In vitro and in vivo correlates of antibody dependent enhancement of EIAV resulting from baculovirus-expressed glycoprotein subunit vaccines. Ann. Mtg. NIH Lab. Tumor Cell Biol. AIDS Res. Human Retroviruses.

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F. APPENDIX :

TABLES 1 - 6
FIGURES 1 - 8

Table 1. Characterization of antibody responses elicited by experimental immunizations with different EIAV envelope immunogens. [a]

Vaccine and Pony No.		Clinical[b] Responses	Antibody Titers[c] rgp90	vgp	Conformational[d] Dependence	Avidity[e] (%)	Neutralization[f] Titer
Immature Immunity (#561)[g] 21 days p.i.			**ND**	**6000**	**0.6**	**≤5**	**N.R.**
Mature Immunity (#561) 301 days p.i.			**ND**	**500000**	**1.5**	**50**	**3860**
1991 rgp90	1-5	Severe	1600	800	0.8	≤5	N.R.
	1-6	Severe	3200	400	0.8	≤5	N.R.
	1-7	Severe	1600	50	0.7	≤5	N.R.
	21	Severe	3200	50	0.4	≤5	N.R.
1995 rgp90	526	Severe	51200	6400	0.7	≤5	N.R.
	66	Severe	51200	6400	0.7	8	N.R.
	90	Severe	102400	6400	0.6	≤5	N.R.
	65	Severe	102400	6400	0.7	≤5	N.R.
	60	Typical	51200	6400	0.7	≤5	N.R.
	120	Typical	102400	3200	0.7	8	N.R.
	118	Typical	102400	6400	0.7	≤5	N.R.
	64	Typical	51200	3200	0.7	≤5	N.R.
	73	Asymptomatic	12800	1600	0.6	14	N.R.
	56	Asymptomatic	51200	3200	0.6	≤5	N.R.
1995 LLgp	45	Asymptomatic	3200	102400	1.2	9	N.R.
	84	Asymptomatic	6400	102400	1.2	8	N.R.
	95	Asymptomatic	12800	102400	1.0	≤5	N.R.
	100	Asymptomatic	6400	102400	1.3	7	N.R.
1995 vgp90	88	Severe	6400	12800	0.6	≤5	N.R.
	449	Typical	6400	6400	0.5	≤5	N.R.
	412	Typical	3200	6400	0.6	≤5	N.R.
	448	Asymptomatic	6400	12800	0.5	≤5	N.R.
	115	Asymptomatic	6400	6400	0.5	≤5	N.R.
	516	Asymptomatic	3200	3200	0.5	≤5	N.R.

Table 1. (Continued)

[a] All assays are described in detail in Hammond et al. 1997.

[b]Evaluation of clinical responses in immunized ponies compared to nonimmunized controls with respect to appearance and severity of clinical symptoms (fever, diarrhea, etc.) and thrombocytopenia. Severe describes acceleration of fever and thrombocytopenia; typical would describe fever and thrombocytopenia responses similar to non-immunized ponies infected with virus; asymptomatic describes the absence of detectable fever or thrombocytopenia. c.f. Table 2 and Table 6.

[c]End point titers of immune serum reactivity against either the rgp90 antigen or viral envelope glycoproteins (vgp) in Con A-ELISA. (ND = Not Done).

[d]Conformational dependence is defined as the ratio of serum reactivity to native and denatured EIAV envelope proteins as measured in Con A-ELISA. Values greater than 1 indicate a predominance of conformationally dependent antibodies while values of less than 1 indicate predominant reactivity to linear envelope determinants.

[e]Avidity is defined as the resistance of serum antibody-EIAV envelope proteins to treatment with 8M urea in the Con A-ELISA. Values $\leq$30% are considered low avidity, >30% - $\leq$50% are considered intermediate avidity, and >50% are designated as high aviditiy.

[f]Serum neutralization activity is presented as the reciprocal of the serum dilution producing a 50% reduction in EIAV infectivity. N.R. (No Reduction) designates the lack of detectable serum neutralization activity at a 1:2 dilution.

[g]Serum samples taken at 21 dpi. and 30 dpi. in ponies experimentally infected with $EIAV_{PV}$, representing immature and mature immune responses respectively, as defined in Hammond et al 1997.

Table 2 Clinical responses and plasma viremia in naive and rgp 90 immunized ponies infected intravenously with 2.5-3 $\log_{10}$ median infective doses of the pony virulent strain of EIAV

Pony Number	First Fever[a] (dpi)	Platelet Decline[b] (dpi)	Plasma Viremia First Day[c]	Plasma Viremia Number of Days[d]	Clinical Responses[f] Typical Peptide
Naive					
80	None	None	12	10	Typical
98	21	None	8	14	Typical
06	19	None	13	9	Typical
86	None	None	13	9	Typical
49	***18***	***36***	***11***	***10***	***Typical***
87	***35***	***None***	***13***	***9***	***Typical***
561	17	None	11	11	Typical
562	17	None	11	11	Typical
564	18	34	12	10	Typical
567	18	39	11	11	Typical
Immunized[e]					
526	12	15	11	11	Severe
66	20	26	13	9	Severe
90	23	24	10	12	Severe
65	19	22	10	10	Severe
60	31	39	20	1	Typical
120	37	41	37	0	Typical
118	36	37	36	0	Typical
64	37	None	20	1	Typical
73	None	None	12	10	Asymptomatic
56	None	None	None	0	Asymptomatic

(a) Rectal temperature in excess of 103^0.

(b) platelet count of $\leq$ 105,000 per microliter.

(c) Days post-inoculation (dpi). Plasma samples were initially tested undiluted in duplicate flasks of fetal equine kidney cell cultures.

(d) Number of positive assays for plasma viremia detected during first 21 days post challenge with $EIAV_{pv}$.

(e) Immunized ponies were challenged 24 days after their third dose of immunogen (66 days after the first dose).

(f) Overall evaluation of disease severity based on clinical impressions (lethergy, edema, diarrhea, etc.) and hematologic abnormalities.

Table 3 Pathologic gross and microscopic findings on ponies infected with $EIAV_{pv}$

Pony Number	History	Euthanized on Day	Major Findings
526	Immunized/ Enhanced	26	Petechial hemorrhages on mucosal surface of large colon and pleural surfaces of lung Numerous pigment-laden macrophages in circulation Hepatitis, nonsuppurative, periportal and lobular, moderate Vacuolar degeneration of hepatocyptes Moderate to severe hemosiderosis, liver Bone marrow was hypercellular
66	Immunized/ Enhanced	34	Petechial hemorrhages present on mucosa of tongue and lips, pleural surface of lungs and serosa and mucosa of small intestine Hemosiderophages present within circulation Hepatitis, nonsuppurative, periportal, mild Vacuolar degeneration of hepatocytes Mild hemosiderosis, liver Bone marrow was normocellular
90	Immunized/ Enhanced	43	Hepatitis, nonsuppurative, periportal and lobular, mild Mild hemosiderosis, liver
60	Immunized/ Typical	43	Lymphoplasmacytic infiltrate, periportal, liver, mild Vacuolar degeneration of hepatocytes, liver
120	Immunized/ Typical	43	Lymphoplasmacytic infiltrate, periportal, liver mild
73	Immunized/ Asymptomatic	43	Lymphoplasmacytic infiltrate, periportal, liver, very mild
49	Naive/ Typical	43	Petechia hemorrhages in colon and small intestine Mild hemosiderosis, liver Hepatitis, nonsuppurative, periportal and lobular, mild Vacuolar degeneration of hepatocytes Bone marrow as moderately hypercellular

Table 4. Kinetics of virus replication in rgp90-vaccinated and control ponies challenged with $EIAV_{PV}$.

Pony Number	Clinical[a] Status	Day of First[a] First Fever	Viral RNA Levels (Molec. per ml) /*Plasma Viremia*[b] Day 7	Day 14	Day 21
#1-5	Enhanced	13	$<10^2$	$2X10^8$	$1X10^8$
#1-6	Enhanced	15	Neg	$2X10^6$	$1X10^5$
#1-7	Enhanced	13	Neg	$7X10^7$	$4X10^6$
#21	Enhanced	13	Neg	$6X10^6$	$3X10^8$
#526	Enhanced	12	Neg / *Neg*	$2X10^7$ / *Pos*	$2X10^7$ / *Pos*
#66	Enhanced	20	Neg / *Neg*	$2X10^5$ / *Pos*	$2X10^8$ / *Pos*
#90	Enhanced	23	Neg / *Neg*	$2X10^4$ / *Pos*	$2X10^7$ / *Pos*
#65	Enhanced	19	Neg / *Neg*	$1X10^5$ / *Pos*	$2X10^7$ / *Pos*
#60	Typical	31	Neg / *Neg*	$<10^2$ / *Neg*	$6X10^3$ / *Neg*
#64	Typical	37	Neg / *Neg*	$2X10^3$ / *Neg*	$3X10^5$ / *Neg*
#118	Typical	36	Neg / *Neg*	$<10^2$ / *Neg*	$<10^2$ / *Neg*
#120	Typical	37	Neg / *Neg*	$3X10^4$ / *Neg*	$3X10^2$ / *Neg*
#56	Asymptomatic	None	Neg / *Neg*	$<10^2$ / *Neg*	$9X10^2$ / *Neg*
#73	Asymptomatic	None	Neg / *Neg*	$3X10^6$ / *Pos*	$1X10^6$ / *Pos*
#87	Control	35	Neg / *Neg*	$4X10^3$ / *Pos*	$2X10^5$ / *Pos*
#49	Control	18	Neg / *Neg*	$4X10^3$ / *Pos*	$5X10^5$ / *Pos*

[a]As described in Table 2.

[b]Viral RNA molecules per ml were calculated by semiquantitative RT-PCR. Plasma viremia (positive or negative) (c.f. Table 2.) are indicated for comparison.

Table 5. Antigen specificity of serum antibodies after sequential immunization of ponies with EIAV rgp90.

		Vaccine Dose		
Pony Number	**ELISA**	**Third**	**Fourth**	**Fifth**
46	rgp90	1:51,200	>1:51200	Challenged
	vgp	1:25,600	1:3200	
63	rgp90	1:51,200	1:12,800	1:12,800
	vgp	1:6400/12,800	1:1600	1:3200
67	rgp90	1:12,800	1:12,800	1:51200
	vgp	1:25,600	1:1600	1:6400
75	rgp90	1:6400	1:12,800	1:25,600
	vgp	1:6400	1:1600	1:3200
119	rgp90	1:12,800	1:25,600	Challenged
	vgp	1:6400	1:1600	
122	rgp90	1:3200	1:25,600	1:51,200
	vgp	1:6400	1:1600	1:3200

Table 6. Kinetics of virus replication in LLgp-vaccinated, vgp90-vaccinated and naive ponies challenged with $EIAV_{PV}$.

Pony Number	Clinical[a] Status	Day of First[a] First Fever	Viral RNA Levels (Molec. per ml) /*Plasma Viremia*[b] Day 7	Day 14	Day 21
1995 llgp					
#100 (PV)	Asymptomatic	None	Neg / *Neg*	Neg / *Neg*	Neg / *Neg*
#45 (PV)	Asymptomatic	None	ND / *Neg*	Neg / *Neg*	Neg / *Neg*
#95 (PV)	Asymptomatic	None	Neg / *Neg*	Neg / *Neg*	Neg / *Neg*
#84 (PV)	Asymptomatic	None	Neg / *Neg*	Neg / *Neg*	Neg / *Neg*
1995 vgp90					
#449 (PV)	Typical	8	Neg / *Neg*	$7X10^2$ / *Neg*	$1X10^3$ / *Pos*
#412 (PV)	Typical	8	Neg / *Neg*	$1X10^5$ / *Pos*	$<10^2$ / *Neg*
#88 (PV)	Enhanced	8	Neg / *Neg*	$5X10^6$ / *Pos*	$3X10^7$ / *Pos*
#115 (PR)	Asymptomatic	None	Neg / *Neg*	Neg / *Neg*	Neg / *Neg*
#516 (PR)	Asymptomatic	3?	Neg / *Neg*	Neg / *Neg*	Neg / *Neg*
#448 (PR)	Asymptomatic	3?	Neg / *Neg*	Neg / *Neg*	Neg / *Neg*
#87	Control	35	Neg / *Neg*	$4X10^3$ / *Pos*	$2X10^5$ / *Pos*
#49	Control	18	Neg / *Neg*	$4X10^3$ / *Pos*	$5X10^5$ / *Pos*

[a]As described in Table 2.
[b]Viral RNA molecules per ml were calculated by semiquantitative RT-PCR. Plasma viremia (positive or negative) (c.f. Table 2.) are indicated for comparison.

Figure Legends

Figure 1. Reactivity of serum antibodies in rgp90 immunized ponies in Con A-ELISA against the vaccine immunogen (rgp90; dark bars) and against viral envelope proteins (vgp; light bars). (c.f. Table 1) Procedures for Con A-ELISA are described in Hammond et al. 1997.

Figure 2. Profiles of clinical responses (fever and platelet reduction) and development of plasma viremia in rgp90-vaccinated ponies and two naive ponies (#49, #87) after $EIAV_{PV}$ challenge. Plasma viremias were assayed daily during the first 21 dpi. to monitor virus replication prior to EIAV p26 seroconversion.

Figure 3. Profiles of clinical responses and development of plasma viremia to EIV_{PV} challenge in a rgp90-vaccinated donor mare (#102) and her foal (#102F) after passive transfer of immune plasma. All measurements were as described in Figure 1.

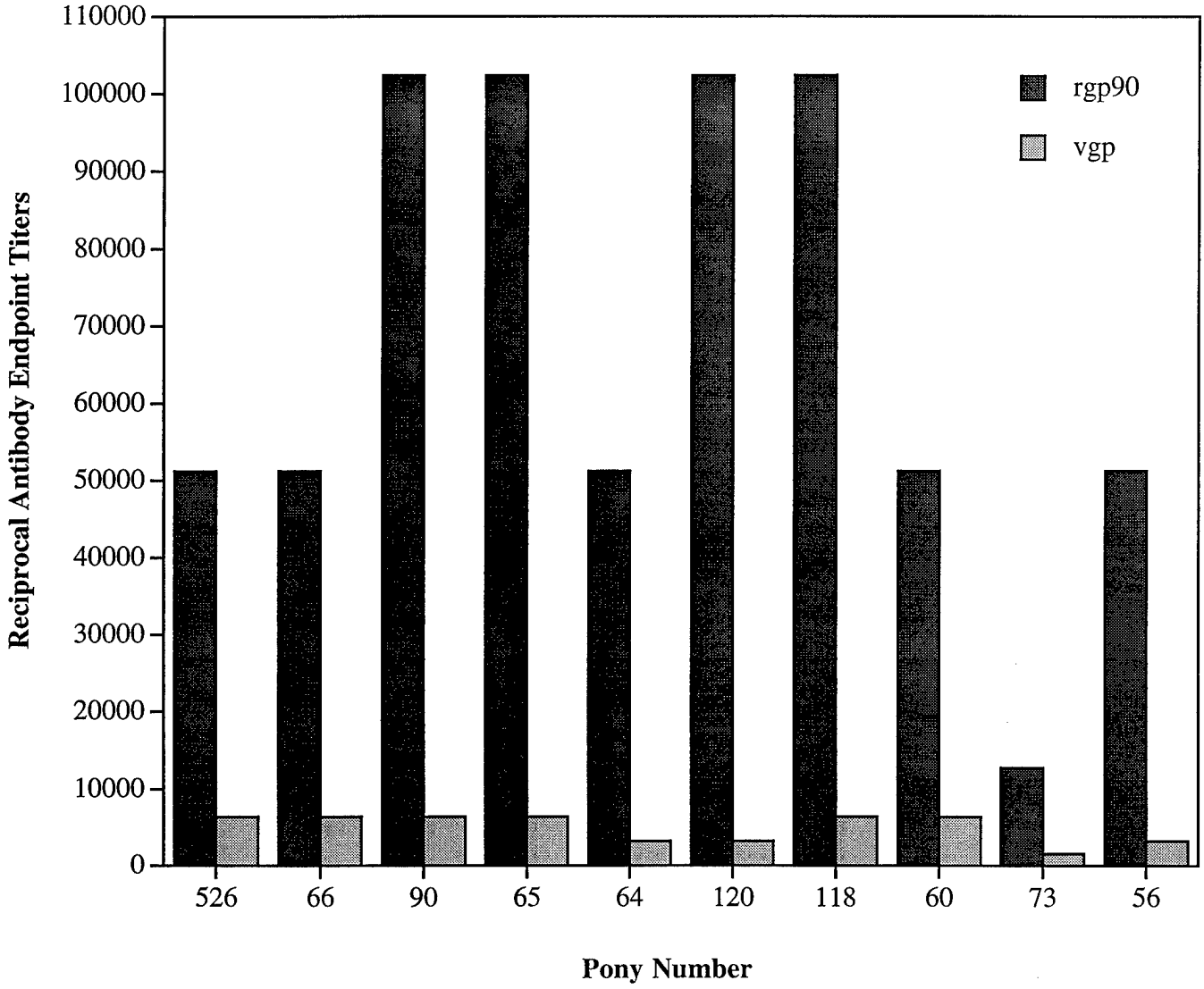
Figure 4. Profiles of clinical responses and development of plasma viremia to $EIAV_{PV}$ challenge of rgp90-immunized donor ponies (#526 and #56) and recipient ponies (#582 and #584, respectively) after passive transfer of immune plasma.

Figure 5. Antigen specificity of serum antibodies in ponies that were sequentially immunized with EIAV rgp90. Reactivity of serum antibodies were evaluated by Con A-ELISA against the vaccine immunogen (rgp90; dark bars) and against viral envelope proteins (vgp; light bars).

Figure 6. Profiles of clinical responses and development of plasma viremia in LL-gp-immunized ponies after homologous challenge with $EIAV_{PV}$.

Figure 7. Profiles of clinical responses and development of plasma viremia in vgp90-immunized ponies after heterologous virus challenge ($EIAV_{PV}$) or homologous virus challenge ($EIAV_{Pr}$).

Figure 8. Antigen specificity of serum antibodies to rgp90 and vgp antigens in Con A-ELISA of immune serum from ponies immunized with rgp90, LL-gp, or vgp90 vaccines. C.f. Table 1.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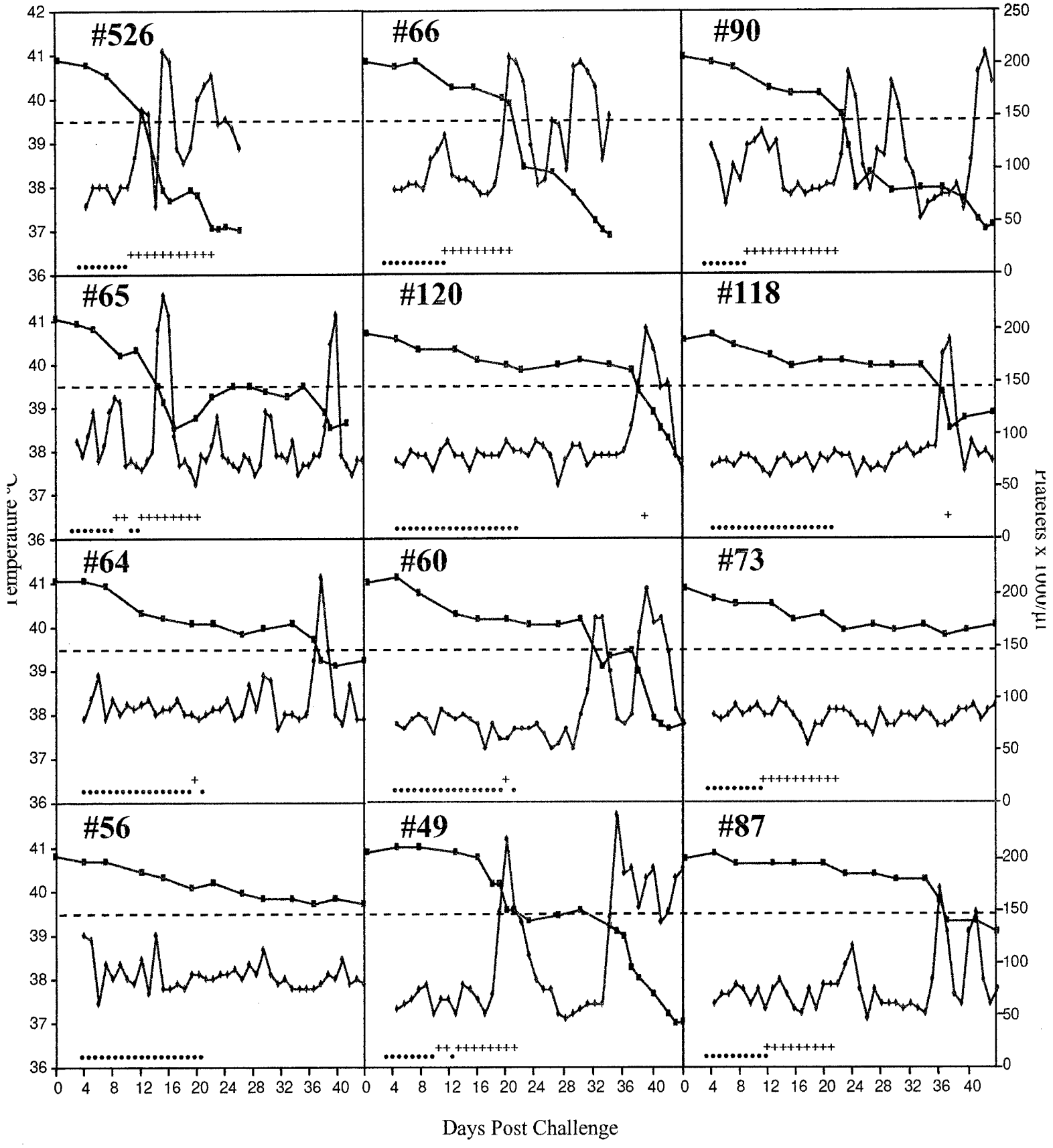
60000
50000
40000
30000
20000
10000
0
Reciprocal Antibody Endpoint Titers
rgp90
vgp
526
66
90
65
64
120
118
60
73
56
Pony Number

Figure 1.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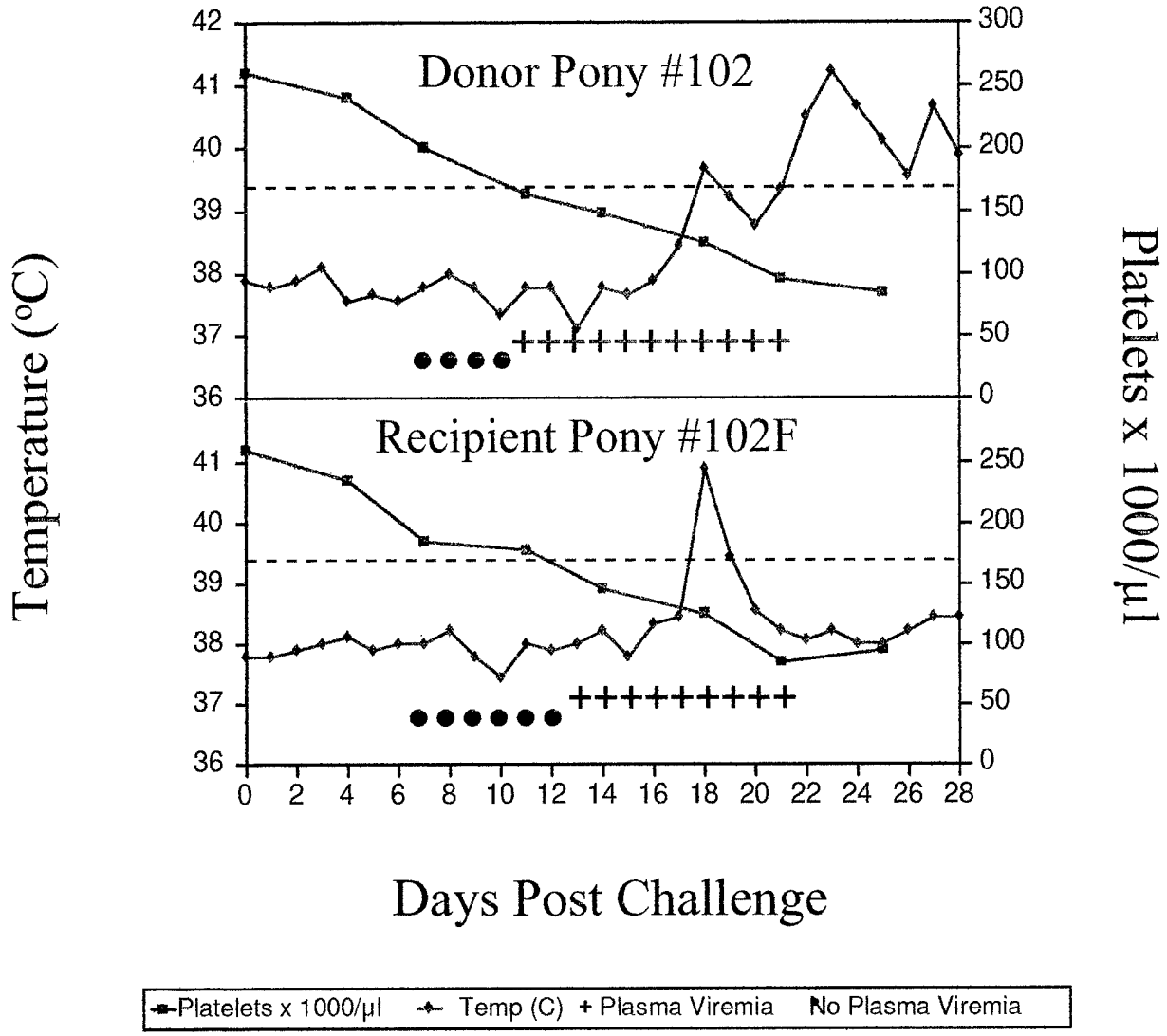
#65
#120
#118
#64
#60
#73
#56
#49
#87
Temperature °C
Platelets x 1000/µl
Days Post Challenge
Temp (C) Platelets x 1000/µl + Plasma Viremia • No Plasma Viremia

FIGURE 2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Recipient Pony #102F
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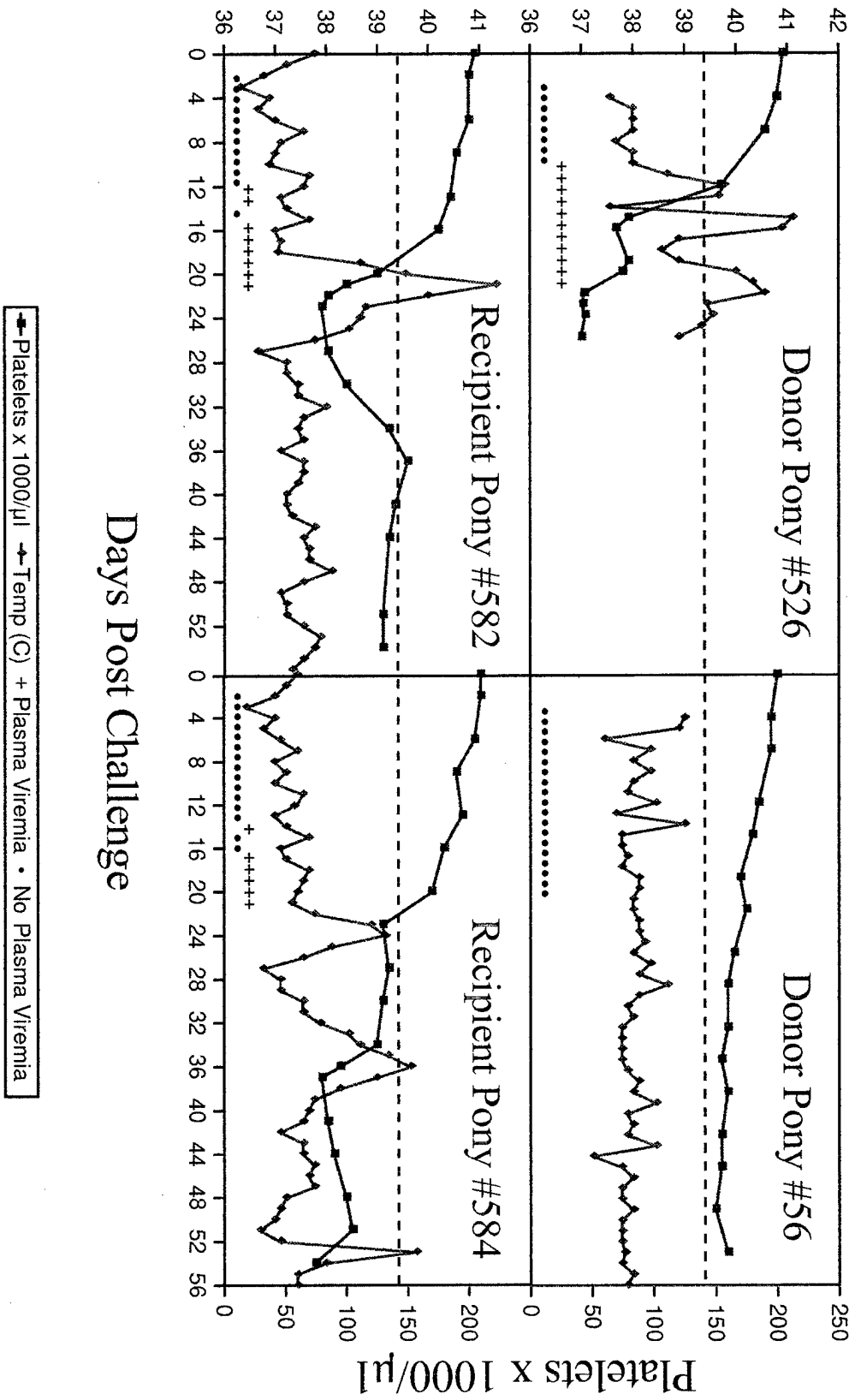
Days Post Challenge
Platelets x 1000/µl
Temp (C)
+ Plasma Viremia
No Plasma Viremia

FIGURE 3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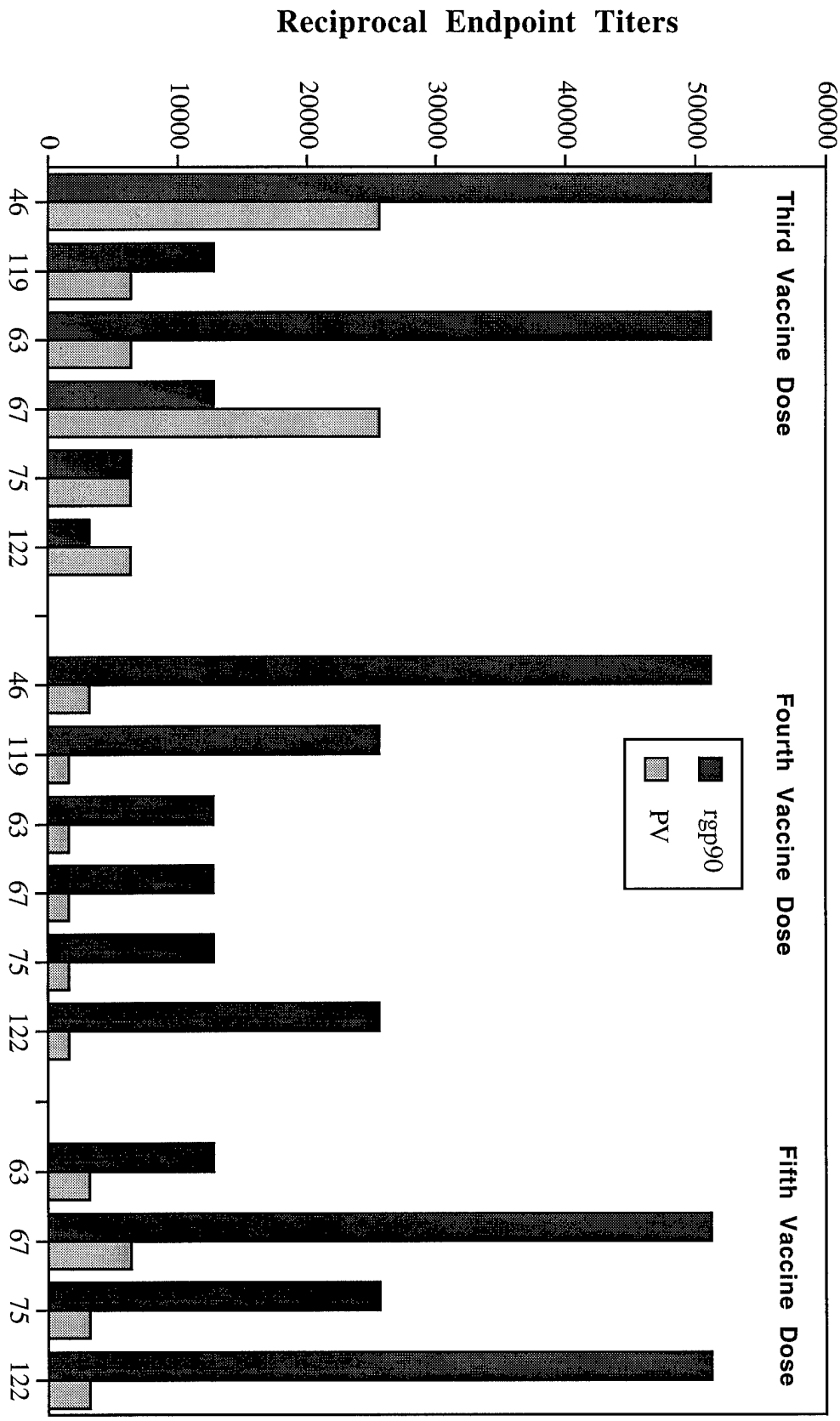
Donor Pony #56
Recipient Pony #582
Recipient Pony #584
Platelets x 1000/µl
Days Post Challenge
Platelets x 1000/µl
Temp (C)
Plasma Viremia
No Plasma Viremia

FIGURE 4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50000
40000
30000
20000
10000
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Third Vaccine Dose
Fourth Vaccine Dose
Fifth Vaccine Dose
rgp90
PV
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119
63
67
75
122
46
119
63
67
75
122
63
67
75
122
Pony Number

Figure 5

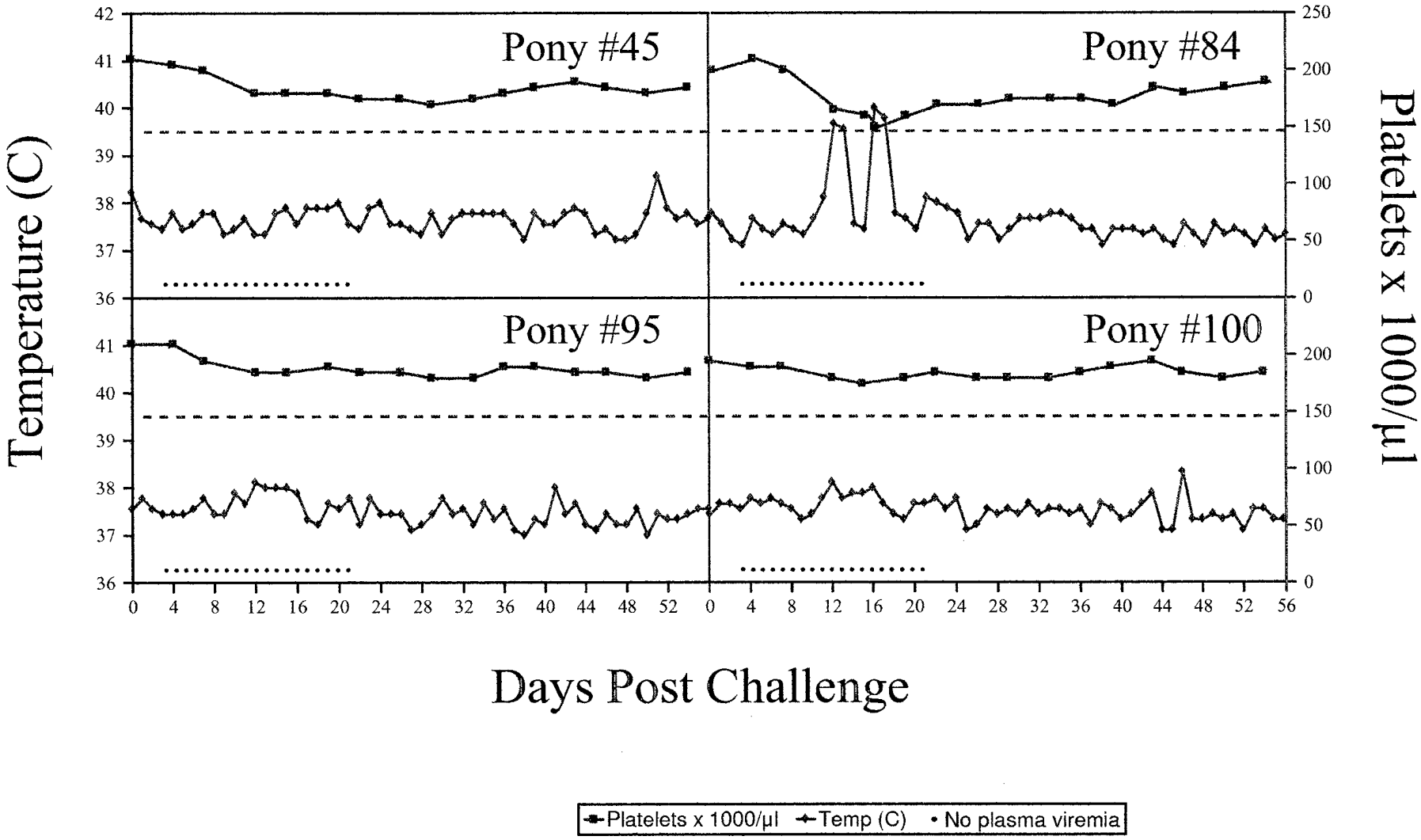

Pony #45
Pony #84
Pony #95
Pony #100
Temperature (C)
Platelets x 1000/µl
Days Post Challenge
Platelets x 1000/µl
Temp (C)
No plasma viremia

FIGURE 6
42

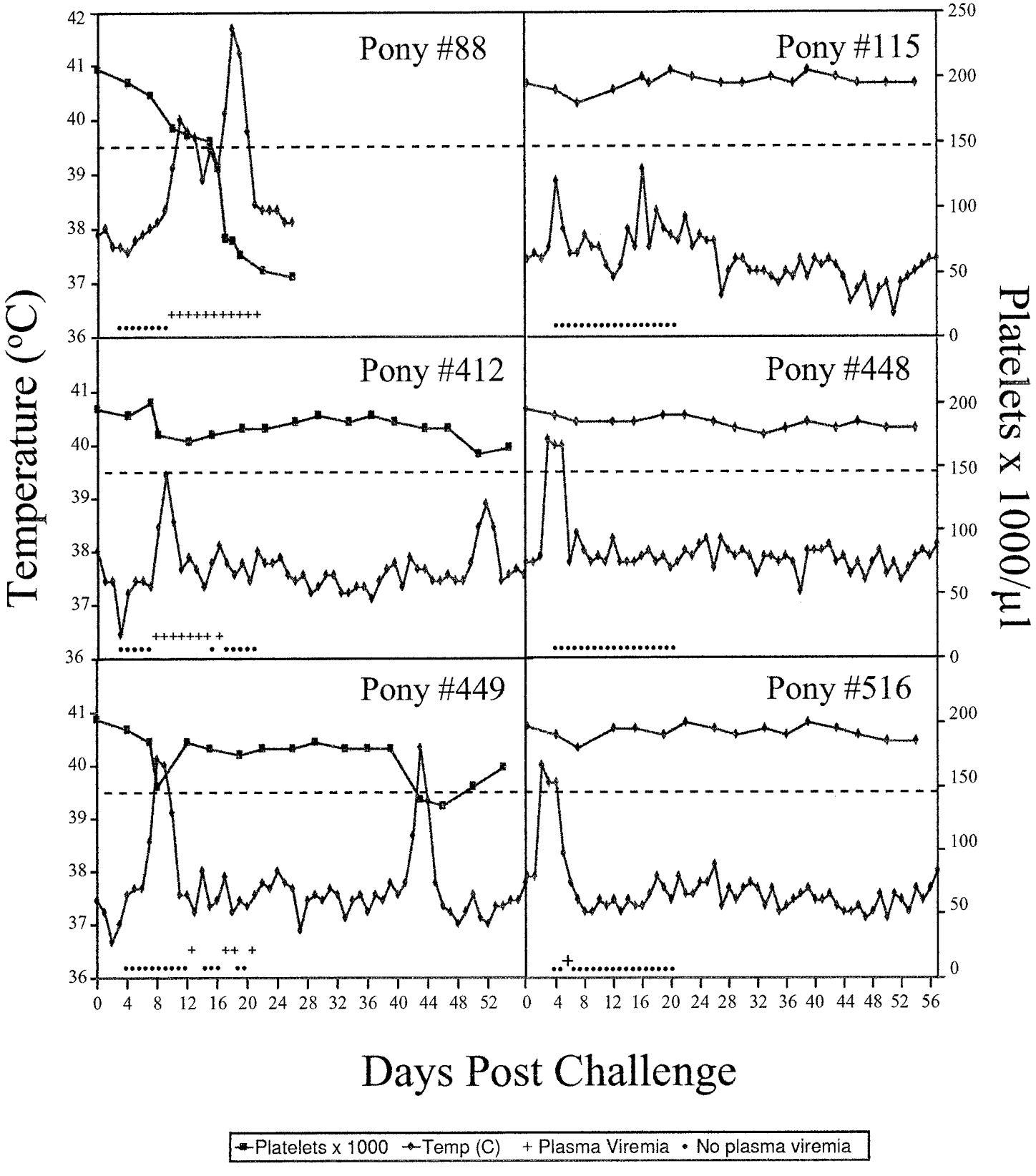
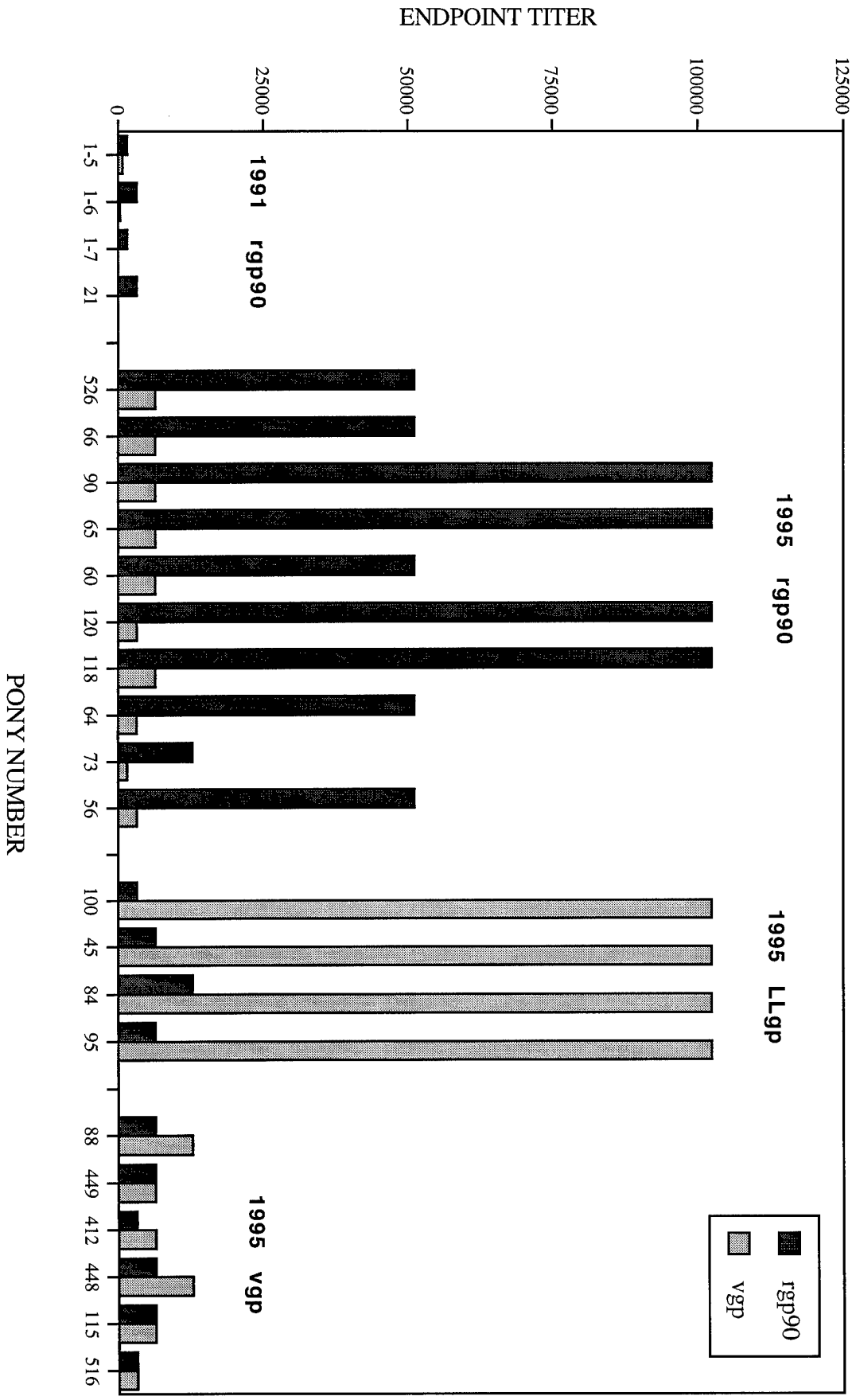

Pony #88
Pony #115
Pony #412
Pony #448
Pony #449
Pony #516
Temperature (°C)
Platelets x 1000/µl
Days Post Challenge
Platelets x 1000
Temp (C)
+ Plasma Viremia
• No plasma viremia

FIGURE 7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1991 rgp90
1995 rgp90
1995 LLgp
1995 vgp
rgp90
vgp
1-5 1-6 1-7 21 526 66 90 65 60 120 118 64 73 56 100 45 84 95 88 449 412 448 115 516
PONY NUMBER

Figure 8